

## INTRANASAL INFECTION OF MONKEYS WITH JAPANESE ENCEPHALITIS VIRUS: CLINICAL RESPONSE AND TREATMENT WITH A NUCLEASE-RESISTANT DERIVATIVE OF POLY(I)·POLY(C)\*

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**Abstract.** In the first experiment two rhesus (*Macaca mulatta*) and two cynomolgus (*Macaca fascicularis*) monkeys were inoculated intranasally (i.n.) with  $3 \times 10^7$  plaque-forming units (PFU) of the Peking strain of Japanese encephalitis virus (JEV) to establish the time course of infection and resulting mortality. The onset of clinical signs for both species of monkeys occurred on days 5 to 9, with fever of several days duration, anorexia and depression. Death ensued in 11 to 12 days. An i.n. median lethal dose equivalent to  $2.5 \times 10^4$  PFU of the Peking strain of JEV was determined in 16 additional cynomolgus monkeys. Clinical signs of infection, virus-neutralizing antibody formation, and mortality were dose-dependent for the doses of virus inoculated. Total peripheral blood leukocyte and neutrophil values increased midway during the course of infection in monkeys that died with encephalitis. Microscopic lesions of JE were similar in monkeys that died following virus challenge. No species-related differences in response to JEV challenge were evident. A nuclease-resistant complex of polyribonucleosinic-polyribocytidylic acid, poly-L-lysine and carboxymethylcellulose [poly(ICLC)] reduced mortality by 50% in monkeys treated initially 8 or 24 h after virus challenge. Mean survival time of nonsurvivors was prolonged 3.5 days and microscopic lesions of encephalitis were less severe in the poly(ICLC)-treated monkeys when compared to infected-untreated monkeys. The response of rhesus and cynomolgus monkeys to JEV challenge by the i.n. route of inoculation thus provides a useful model for the study of potential antiviral compounds in host defense against Japanese encephalitis.

Previous studies have shown that interferon may play a role in protecting animals against Japanese encephalitis virus (JEV).<sup>1</sup> Mice treated with a synthetic inducer of interferon, the polyribonucleotide complex of inosinic and cytidylic acids, poly(I)·poly(C),<sup>2</sup> were partially protected from challenge with JEV if drug treatment was begun up to 24 h postinfection.<sup>1</sup> In contrast, poly(I)·poly(C) was not an effective inducer of interferon in subhuman primates, apparently because of rapid enzymatic degradation of the compound in primate serum.<sup>3</sup> However, a nuclease-resistant complex of poly(I)·poly(C), poly-L-lysine, and carboxymethylcellulose [poly(ICLC)] induces high levels of interferon in both monkeys and chimpanzees.<sup>3-5</sup> It has been used successfully

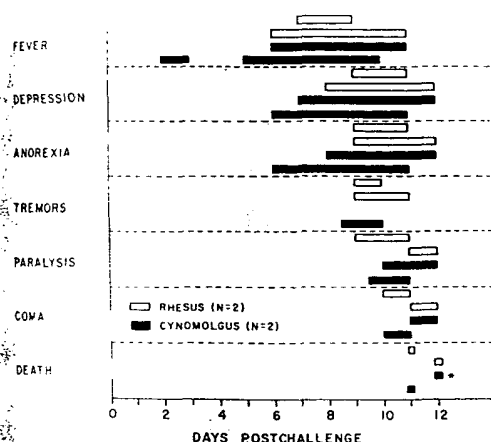
to protect rhesus monkeys against both simian hemorrhagic fever<sup>3</sup> and yellow fever.<sup>6</sup> Poly(ICLC) has not been employed against any other experimental flavivirus infections which may be sensitive to interferon.

Experimental infection of monkeys and mice with JEV by the intracranial (i.c.)<sup>7</sup> and intranasal (i.n.)<sup>8-10</sup> routes of inoculation were first reported by Japanese workers several decades ago. They reported clinical signs of infection and pathologic lesions of encephalitis in monkeys similar to those described for fatal cases of JE in man.<sup>7-14</sup> Monkeys experimentally infected with JEV by peripheral routes other than i.n. frequently experience an immunizing infection with viremia but without clinical evidence of encephalitis.<sup>15,16</sup> The i.n. route of inoculation causes fatal JEV disease in monkeys without direct inoculation of virus into the central nervous system (CNS).<sup>17,18</sup>

The purpose of this study was to (a) characterize experimental JE in rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys

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\* Euthanasia was performed on monkey when moribund.

FIGURE 1. Onset, duration, and frequency of clinical signs in two rhesus and two cynomolgus monkeys inoculated intranasally with  $3 \times 10^7$  plaque-forming units of the Peking strain of Japanese encephalitis virus.

( $1.5 \times 10^6$  PFU). Subsequently, the monkeys were allocated equally into three groups: poly(ICLC) treatment initiated either 8 or 24 h following virus challenge, and JEV-inoculated, untreated controls. All drug-treated monkeys received 0.3 mg/kg of poly(ICLC) intravenously once each day beginning either 8 or 24 h post-challenge and on days 5 to 9, whereas on days 2 to 4, the dosage was 3.0 mg/kg. Infected-untreated control monkeys received sham treatment once daily with an equivalent volume of pyrogen-free saline. Sera collected prior to virus inoculation and at periodic intervals after infection were assayed for JEV SN antibody.

**Hematology.** Blood samples for white blood cell (WBC) counts were diluted with the Unopette® System (Beckton-Dickinson and Co., Rutherford, NJ) and leukocytes were counted in a hemacytometer. Differential counts were made from Wright's stained blood smears.

**Histopathology.** Necropsy was performed on monkeys shortly after death; tissues from all organ systems were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 4–6  $\mu$  and stained with hematoxylin and eosin. Neural tissues were sectioned at the lumbar and cervical spinal cord, medulla, cerebellum, cerebral cortex, thalamus, and olfactory lobes for examination.

**Calculations.** The i.n. LD<sub>50</sub> was calculated by probit analysis<sup>21</sup> using survival data from study 2.

Student's *t*-test was used for intergroup comparisons of mean rectal temperature and hematologic data. Significant differences are noted when  $P < 0.05$ .

## RESULTS

### Study 1: Response of rhesus and cynomolgus monkeys challenged with $3 \times 10^7$ PFU of JE (Peking) virus

Time of onset, frequency and duration of clinical signs, and time-to-death for these monkeys following i.n. virus challenge are shown in Figure 1. Clinical signs of infection appeared in the same general sequence in each monkey: fever ( $>39.5^\circ\text{C}$ ), depression, anorexia, tremors, paralysis, coma, and death (Fig. 1). The time of onset and duration of clinical signs were similar for both species. One cynomolgus monkey experienced a brief febrile response on day 2 whereas all other monkeys were febrile between days 5 and 7. The mean peak febrile response occurred on day 7 ( $40.4^\circ\text{C}$ ) in rhesus and on day 8 ( $40.2^\circ\text{C}$ ) in cynomolgus monkeys. Depression and anorexia, mild at onset and associated with fever, progressed in severity until death. The duration of febrile response was similar for both species. Rectal temperature dropped precipitously ( $<34^\circ\text{C}$ ) in all monkeys beginning 24 to 48 h prior to death. Three of the four monkeys developed slight to moderate muscular tremors of the head, trunk, and extremities by day 9. Ataxia and partial spastic paralysis of the extremities were followed in turn by inability to sit or stand, complete paralysis, and coma. The rhesus monkeys died on days 11 and 12 postchallenge, whereas one cynomolgus monkey died on day 11 and euthanasia was performed on the other when moribund on day 11. The rhesus and one cynomolgus monkey had JEV SN antibody titers of 1:10 on day 10. The other two monkeys died before SN antibody was detected.

### Study 2: Intranasal LD<sub>50</sub> determination

Seven of 16 cynomolgus monkeys challenged i.n. with JEV developed fever and encephalitis, and died (Table 1). These included all five monkeys in the highest dose group ( $1 \times 10^5$  to  $10^6$  PFU), 2 of 6 in the intermediate group ( $4 \times 10^3$  to  $10^4$  PFU), and none of 5 in the low ( $4 \times 10^1$  to  $10^2$  PFU) virus-dose group (Table 1). The monkey i.n. LD<sub>50</sub> was  $2.5 \times 10^4$  PFU. Time-to-death was

inoculated by the i.n. route with JE (Peking) virus; and (b) evaluate the potential effectiveness of poly(ICLC) in the therapeutic management of JE in nonhuman primates.

#### MATERIALS AND METHODS

**Virus.** Stock virus was prepared from a 5th suckling mouse brain passage of the Peking strain of JEV.<sup>19</sup> A 50% stock suspension containing  $8 \times 10^8$  plaque-forming units (PFU) of virus/ml was prepared in 50% normal, inactivated, fetal bovine serum infusion broth and maintained at  $-70^\circ\text{C}$  until used. Inocula were prepared by diluting the stock virus suspension in phosphate-buffered saline containing 1% heat-inactivated rabbit serum. Assays for virus in inocula were performed by a modification of the viral plaque assay technique of Rhim.<sup>20</sup> Serial 10-fold dilutions of inocula were made in Hank's balanced salt solution (HBSS) containing 25 mM Hepes buffer and 5% IFBS. Two-tenths milliliter of each virus dilution was inoculated onto monolayer cultures of baby hamster kidney (BHK-21) cells grown in plastic trays (9.6 cm<sup>2</sup>/well, of Limbro Scientific Co. Inc., New Haven, Conn.). After 1.5 h incubation at  $36^\circ\text{C}$  in a humidified atmosphere containing 5% CO<sub>2</sub>, samples were overlaid with 3 ml of maintenance medium<sup>20</sup> in 1% agarose. Four days later an additional 1.5 ml of overlay medium containing 1:5,000 w/v of neutral red was added. Plaques were counted 24 h after the second overlay. All assays were done in triplicate.

**Neutralizing antibody assay.** JEV serum neutralizing (SN) antibody was assayed on BHK-21 cells grown in 6-well plastic trays (9.6 cm<sup>2</sup>/well). A mixture of 0.5 ml of virus containing approximately  $10^6$  PFU/0.1 ml and 0.5 ml of various serum dilutions was incubated at  $36^\circ\text{C}$  for 1.5 h, then 0.2 ml of the mixture was inoculated per well. After 1.5 h incubation at  $36^\circ\text{C}$  the double agar overlay was added as described above. Plaques were counted on the 5th day of incubation with 80% plaque-reduction selected as the end-point. All assays were done in triplicate.

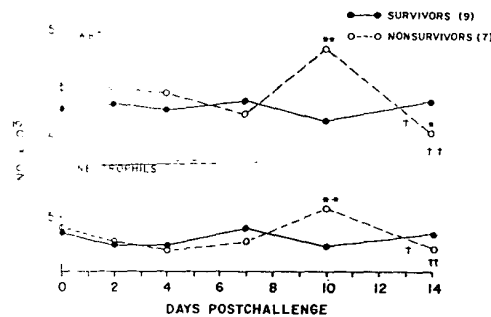
**Poly(ICLC).** Poly(ICLC) was prepared at the National Institutes of Health as previously described.<sup>1</sup> The final solution contained 2 mg of poly(I), poly(C), and bound as the complex with poly-L-lysine and carboxymethylcellulose. The complex was stored at  $4^\circ\text{C}$  and diluted in an equal volume of pyrogen free saline prior to use.

**Monkeys.\*** Healthy, well-conditioned, adult monkeys of both sexes, (8 rhesus and cynomolgus) weighing 3.6 to 4.9 and 1.9 to 2.5 kg, respectively, were used. All monkeys were negative ( $<1:5$ ) for JEV serum neutralizing antibody and negative ( $<1:10$ ) for JE, Nile, yellow fever and dengue serotypes 1, 2, 3 hemagglutination inhibition antibodies prior to use. Monkeys were housed in individual cages in rooms maintained at constant temperature (23 to 25°C) with a 12-h light cycle. They were fed twice daily with commercial monkey chow (Wayne Monodiet, Allied Mills, Inc., Chicago, Ill.) and provided water ad libitum. Monkeys were injected intramuscularly with 10 mg/kg of Ketaset<sup>®</sup> (ketamine hydrochloride, Bristol Laboratories, Syracuse, N.Y.) for restraint before i.n. inoculation with 0.5 ml of JEV. Half (0.25 ml) of inoculum was given drop-wise into each nostril.

**Experimental design.** Three studies were conducted in which monkeys were inoculated with the Peking strain of JEV. First, two rhesus and two cynomolgus monkeys were inoculated with  $3 \times 10^7$  PFU of JEV to assess their response to i.n. challenge. In the second study, 16 cynomolgus monkeys were divided at random into challenge groups to determine both the response to i.n. JEV challenge and the LD<sub>50</sub>. Groups inoculated with 40 to  $4 \times 10^6$  PFU of 1 strain of JEV in serial 10-fold decrements of stock virus suspension. In both studies, the experimental infection was allowed to follow its natural course. Clinical signs, including rectal temperature, depression, anorexia, tremors, paralysis, coma, were recorded daily. Femoral blood (approximately 3 ml) for hematologic and biologic studies were collected before and after 0, 2, or 4th day following virus challenge.

The third experiment was a preliminary study to evaluate treatment with poly(ICLC) in inoculation with an established nonlethal dose of JEV. Six rhesus monkeys inoculated i.n. with  $10^6$  PFU of JEV were

\* In conducting the research described herein, the investigators adhered to the Guidelines for the Care and Use of Laboratory Animals as promulgated by the Committee on the Revision of the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, and the American Association for Accreditation of Laboratory Animal Care.



† Indicates dead monkey; \*  $P < 0.05$ ; \*\*  $P < 0.025$ .

FIGURE 2. Mean total leukocyte and neutrophil values for nine surviving and seven nonsurviving monkeys inoculated intranasally with serial 10-fold dilutions of the Peking strain of Japanese encephalitis virus. The base-line values represent the mean for survivors and nonsurvivors 2 days prior to virus inoculation.

dose-related in 6 of 7 monkeys that developed clinical signs and died following virus challenge. Clinical signs of JE in these monkeys were similar to those shown in Figure 1. None of the five monkeys in the low virus-dose group were febrile or showed any clinical signs of encephalitis following virus challenge. Mean rectal temperatures for surviving and nonsurviving monkeys were significantly different ( $P < 0.05$ ) on days 8 to 10 and 12 to 16 postchallenge. Mean peak febrile response in the nonsurviving monkeys occurred on day 9 ( $39.8^{\circ}\text{C}$ ), followed on day 12 by a decline in mean rectal temperature late in the course of the infection.

Mean total WBC and absolute neutrophil values were increased significantly ( $P < 0.05$ ) on day 10

in the nonsurviving group (Fig. 2). Subsequent WBC values were decreased in four remaining monkeys on day 14 in the nonsurviving group (Fig. 2). Mean absolute lymphocyte values were significantly ( $P < 0.05$ ) decreased on day 14 in the nonsurviving group compared with the surviving group. Three of seven nonsurviving monkeys in the high and intermediate virus-dose groups developed overt signs of infection and died before SN antibody to JEV was detected. One monkey in the intermediate virus-dose group given  $4 \times 10^3$  PFU of virus showed no apparent clinical signs of illness; however, a JEV SN antibody titer of 1:10 was present on day 14. None of the five monkeys in the low virus-dose group had detectable JEV SN antibody.

Gross and microscopic lesions were similar in rhesus and cynomolgus monkeys which died during studies 1 and 2. On gross examination, all major organ systems appeared essentially normal. Significant microscopic changes of varying severity were observed within sections of brain and spinal cord of each monkey. A meningeal lymphocytic infiltrate was present over all areas of the brain and spinal cord with the most prominent foci being in the cerebellar sulci (Fig. 3). Lymphocytic perivascularitis was commonly found in both the grey and white matter of the brain and spinal cord (Fig. 4). Glial cell proliferation was pronounced in grey matter of the spinal cord, brain stem, and thalamus. Neuronal degeneration, necrosis and neuronophagia (Fig. 5) were not common in spinal cord and medulla, but were present (although less severe) in all CNS sections. Olfactory lobes were normal except for minimal meningeal lymphocytic infiltrates and occasional

TABLE 1  
Clinical findings and serum neutralizing (SN) antibody responses in cynomolgus monkeys following intranasal challenge with the Peking strain of Japanese encephalitis virus (JEV)

Virus-dose group	Virus-dose (PFU)	Fever* (no./total)	No. dead/total	Day of death	Reciprocal JEV antibody titer (day 14)†
High	$4 \times 10^6$	2/2	2/2	14, 15	20, 105
	$4 \times 10^5$	3/3	3/3	15, 15, 17†	20, 5, <5
Intermediate	$4 \times 10^4$	1/3	1/3	1	<5, <5
	$4 \times 10^3$	1/3	1/3	1, 2	<5, <5, 10
Low	$4 \times 10^2$	0/3	0/3		<5, <5, <5
	$4 \times 10^1$	0/2	0/2		<5, <5

\* Rectal temperature  $\geq 39.5^{\circ}\text{C}$ .

† Monkey died with encephalitis before JEV SN antibody was detected.

‡ JEV serum neutralizing antibody inhibiting 80% plaque formation.

§ Day 1 JEV SN antibody titer.

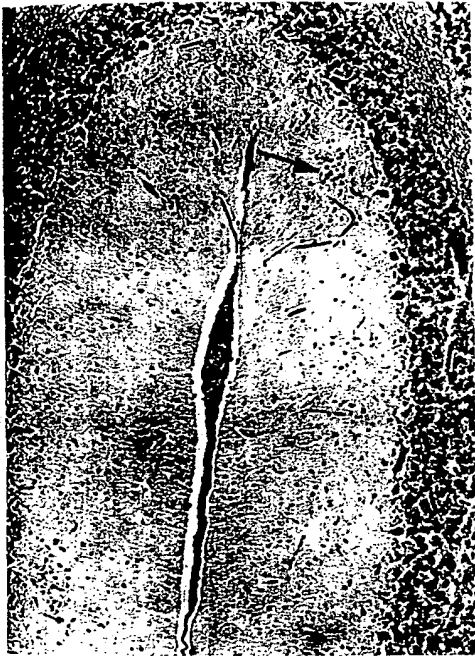


FIGURE 3. Mild lymphocytic infiltration of the meninges in a cerebellar sulcus (center) and small focus of gliosis in the molecular layer (arrow) in a monkey inoculated intranasally with the Peking strain of Japanese encephalitis virus. H & E.  $\times 46$ .

perivascular cuffing. Microscopic lesions were found outside the CNS in some infected animals. Subacute myocarditis was present in two monkeys, one of which had myocardial fiber degeneration. Hemorrhage at the adrenal corticomedullary junction was seen in one monkey and lymphoid follicular degeneration was present in the spleen of several monkeys challenged with JEV.

#### *Study 3: Effect of poly(ICLC) on JEV infection in monkeys*

Two of four monkeys treated with poly(ICLC) following i.n. virus inoculation survived a lethal challenge dose of JEV. Deaths occurred in two poly(ICLC)-treated monkeys on days 18 and 19 postchallenge, one from each of the 8- and 24-h treatment groups (Table 2). Both infected, untreated monkeys died 2 to 5 days earlier than the poly(ICLC)-treated monkeys with predicted courses of infection for the challenge dose of JEV employed (Table 2). All four monkeys that died had clinical courses of infection and histopatho-

logic lesions as described in study 2. However, lesions within the CNS and spleen of poly(ICLC)-treated monkeys were fewer and less severe when compared to lesions in the infected-untreated monkeys.

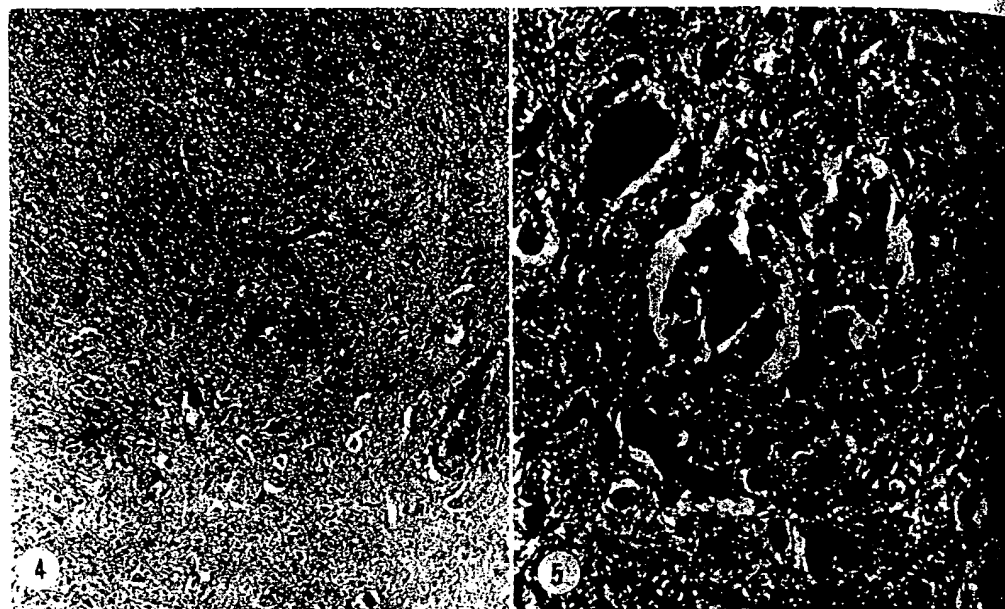
In the poly(ICLC)-treated groups, monkey A had a JEV SN antibody titer of 1:10 on day 18, the day of death, while monkey B was negative throughout the experimental period (Table 2). Monkey C, which died, and monkey D, which survived infection, had JEV SN antibody titers of 1:5 and 1:20, respectively, on day 14 (Table 2). Infected-untreated monkeys E and F died before JEV SN antibody was detectable (Table 2).

#### DISCUSSION

A subhuman primate model was characterized for testing the efficacy of potentially important antiviral compounds for the control of JE in man. The intranasal route of challenge was selected to induce encephalitis without inoculating the virus directly into the CNS. Clinical signs, hemograms, and histopathologic lesions are in agreement with previous reports of natural infections in man,<sup>12-22</sup> and experimental JEV infection in rhesus<sup>10</sup> and Taiwan macaques<sup>14, 15</sup> infected by the i.n. route. Mortality in both rhesus and cynomolgus monkeys was virus-dose dependent and death occurred between 11 and 18 days postchallenge, which is consistent with previous reports of i.n. JEV challenge in macaques.<sup>15</sup> Approximately  $4 \times 10^5$  PFU or more of the Peking strain of JEV was uniformly fatal in nonimmune monkeys.

Glia cell proliferation, neuronal necrosis and neuronophagia, and perivascular lymphocytic infiltration in the leptomeninges, brain, and spinal cord were lesions consistent with those reported from fatal cases of JE in monkeys following i.n. or i.m.<sup>6, 10</sup> challenge and in natural infections of man.<sup>12-23</sup> Studies in mice<sup>8, 9</sup> and monkeys<sup>10, 11</sup> following i.n. inoculation of JEV indicate that virus enters the brain by way of the nasal sinus, first localizing in the olfactory lobes where it multiplies and then invades other areas of the brain and circulatory system.

Poly (ICLC) treatment for 9 consecutive days protected 2 of the 4 rhesus monkeys against a lethal challenge dose of JEV and prolonged the time-to-death of nonsurviving monkeys. Prolonged survival time and reduced severity of



FIGURES 4 and 5. Ventral horn of the spinal cord from a monkey following intranasal inoculation of Japanese encephalitis virus (Peking strain). 4. Perivascular lymphocytic cuffing (top center) and focal gliosis (arrows). H & E,  $\times 50$ . 5. Satellitosis, neuronal necrosis, and neuronophagia (arrows). H & E,  $\times 320$ .

encephalitic lesions in poly(ICLC)-treated monkeys which died indicate a partial reduction in the severity of JE infection by poly(ICLC), even when treatment was initiated 24 h following virus inoculation. To our knowledge, this is the first report which indicates that poly(ICLC), a potent interferon inducer, is beneficial in the early treatment of an otherwise fatal JEV infection in subhuman primates. It is possible that an alternative

regimen of therapy might increase the protective efficacy of poly(ICLC) against JEV infection in monkeys. Protection in poly(ICLC)-treated monkeys cannot be explained by earlier and greater SN antibody than in the infected-untreated controls. Poly(ICLC) did not appear to eliminate infection in all monkeys surviving JEV challenge, as evidenced by SN antibody formation. This is in consonance with previous reports in which

TABLE 2  
*Effect of polyribonucleoside-polyribocytidylic acid poly-L-lysine in rhesus monkey inoculated intranasally with the Peking strain of Japanese encephalitis virus (JEV)*

Time of initial treatment*	Monkey no.	Reciprocal JEV SN antibody titer† by days			Days paralyzed	Day of death
		14	18	28		
+8 h	A	<5	10	D‡	5	18
	B	<5	<5	<5	0	Survived
+24 h	C	5	20	D	5	19
	D	20	20	20	0	Survived
Untreated	E	<5	D		3	16
	F	D			2	14

\* Treated monkeys received 0.3 mg/kg of poly(ICLC) intravenously once daily at 8 or 24 h post-challenge and on days 5 through 9, whereas on days 2 through 4 the dosage was 3.0 mg/kg.

† JEV serum neutralizing (SN) antibody titer inhibiting 80% plaque formation.

‡ Monkey dead, serology not performed.

poly(I)·poly(C) given to mice up to 24 h following JEV inoculation increased protection against fatal encephalitis, but did not eliminate infection in all surviving animals.<sup>1</sup>

Control of an otherwise lethal JEV infection in two monkeys was reported following treatment with a known potent interferon inducer, poly(ICLC).<sup>3-6</sup> The protection afforded monkeys by poly(ICLC) is presumed to have resulted from interferon induction, although this was not determined in the study. Peking<sup>2</sup> and other strains<sup>23</sup> of JEV have been shown to be sensitive to interferon. The successful use of poly(ICLC) in the treatment of simian hemorrhagic fever<sup>3</sup> and yellow fever<sup>6</sup> infections in nonhuman primates are in general agreement with our findings for JEV infections in monkeys. The i.n. challenge model should prove useful for testing other potential antiviral compounds against JEV infection in subhuman primates. Further studies are planned to investigate different regimens of poly(ICLC) therapy.

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